



TITLE:

β -Aryl- β -amino acid aminotransferase from *Variovorax* sp. JH2 is useful for enantioselective β -phenylalanine production

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1 β -Aryl- β -amino acid aminotransferase from *Variovorax* sp. JH2 is useful for
2 enantioselective β -phenylalanine production

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16

17 Abbreviations: BHA, glutamate-1-semialdehyde 2,1-aminomutase from *Bacillus*

18 *halodurans*; CDI, glutamate-1-semialdehyde 2,1-aminomutase from *Corynebacterium*

1 *diphtheriae*; CNE, glutamate-1-semialdehyde 2,1-aminomutase from *Cryptococcus*
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5 2 *neoformans*; FPLC, fast protein liquid chromatography; FRA, aminotransferase class-III
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8 3 from *Frankia* sp.; *e.e.*, enantiomer excess; GITC,
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11 4 2,3,4,6-tetra-*O*-acetyl- β -D-glucopyranosyl isothiocyanate; HPLC, high-performance
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14 5 liquid chromatography; IP, internal peptide; PLP, pyridoxal 5'-phosphate; (*S*)-BAT,
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17 6 (*S*)- β -phenylalanine:2-oxoglutarate aminotransferase; SDS-PAGE, sodium dodecyl
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20 7 sulfate-polyacrylamide gel electrophoresis; SPO, glutamate-1-semialdehyde
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23 8 2,1-aminomutase from *Schizosaccharomyces pombe*.
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1 ABSTRACT

2 A bacterium, *Variovorax* sp. JH2, has an ability to degrade (*S*)- β -phenylalanine
3 stereoselectively. The enzyme involved in the degradation,
4 (*S*)- β -phenylalanine:2-oxoglutarate aminotransferase, was purified to homogeneity from
5 *Variovorax* sp. JH2 and characterized. The enzyme was useful for (*R*)- β -phenylalanine
6 production from racemic β -phenylalanine by enantioselective decomposition of
7 (*S*)- β -phenylalanine through transamination. (*S*)- β -Phenylalanine and
8 (*S*)-3-amino-3-(3-pyridyl)propionate served as good amino-donors in the transamination
9 and 2-oxoglutarate, oxaloacetate, pyruvate, and 1,3-acetonedicarboxylate served as
10 amino-acceptors. The enzyme had a molecular weight of about 72,000 and consisted
11 of two identical subunits. Three internal amino acid sequences (54, 67, and 63
12 residues) were determined and showed homology with glutamate-1-semialdehyde
13 2,1-aminomutases.

14
15 **Key words:** β -Phenylalanine aminotransferase; β -Aryl- β -amino acid aminotransferase;
16 *Variovorax* sp.; Stereoselective synthesis

1. Introduction

β -Phenylalanine (3-amino-3-phenylpropionic acid) is a type of β -amino acid present in several bioactive molecules, such as antibiotics and enzyme inhibitors [1, 2]. Because of its bioactivity, optically active β -phenylalanine and its derivatives are becoming important chiral building blocks for the synthesis of pharmaceutical agents. Enzymes catalyzing the synthesis reaction of β -phenylalanine are one of the potential methods for the preparation of chiral compounds, however, only a few enzymatic methods for the synthesis of optically active β -phenylalanine and its derivatives have been developed [3-7]. In our previous report, the microbial production of optically active β -phenylalanine through stereoselective degradation of racemic β -phenylalanine was reported [8]. Microorganisms were discovered that can catalyze stereoselective β -phenylalanine degradation available for the synthesis of optically active β -phenylalanine, and efficient β -phenylalanine production was achieved using these potent strains. In particular, a potent strain *Variovorax* sp. JH2 isolated as a β -phenylalanine-assimilating microorganism showed an ability to degrade (S)- β -phenylalanine stereoselectively through transamination with 2-oxoglutarate as an amino-acceptor. Some enzymes involved in β -amino acid metabolism have been reported, such as ω -amino acid aminotransferases and leucine 2,3-aminomutase [9-14].

1 However, there are a few reports on β -phenylalanine aminotransferases [6, 7]. The
2 present study investigated the aminotransferase involved in β -phenylalanine metabolism
3 of *Variovorax* sp. JH2. The potential of the purified enzyme for (*R*)- β -phenylalanine
4 production from racemic β -phenylalanine by enantioselective decomposition of
5 (*S*)- β -phenylalanine through transamination in the presence of 2-oxoglutarate was
6 examined and the detailed characterization of the enzyme,
7 (*S*)- β -phenylalanine:2-oxoglutarate aminotransferase ((*S*)-BAT), is described.

2. Materials and methods

2.1. Chemicals

11 DL-3-Amino-3-phenylpropionic acid (racemic β -phenylalanine) was purchased from
12 Aldrich Chemical Co. (Milwaukee, WI, USA). 3-Oxo-3-phenylpropionic acid
13 (benzoylactic acid) was a kind gift from Kaneka Co., Ltd (Osaka, Japan). All other
14 chemicals used in this work were of analytical grade and available commercially.

2.2. Microorganisms and cultivation

17 *Variovorax* sp. JH2 isolated from a soil sample as a β -phenylalanine-assimilating
18 microorganism was used as a source of enzyme. The strain was cultivated at 28°C

1 with shaking (120 strokes/min) for 24 h in 2-L flasks containing 500 ml of medium
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5 2 comprising 0.1% (w/v) KH_2PO_4 , 0.1% (w/v) K_2HPO_4 , 0.03% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$,
6
7
8 3 0.01% (w/v) yeast extract, 0.2% (w/v) NH_4Cl , and 0.3% (w/v) racemic β -phenylalanine
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11 4 (pH 7.0).
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18 2.3 Enzyme assays

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21 7 The standard methods for the activity assay were as follows. The 200 μl reaction
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24 8 mixture contained 10 mM racemic β -phenylalanine, 10 mM 2-oxoglutarate, 5 mM
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27 9 pyridoxal-5'-phosphate (PLP), 200 mM Tris/HCl buffer (pH 9.0), and an appropriate
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31 10 amount of enzyme. The mixture was incubated at 28°C for 15 to 120 min where the
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34 11 reaction proceeded linearly, and the reaction was terminated by adding 20 μl of 15%
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37 12 (v/v) perchloric acid. Investigations of the effects of pH, temperature, and chemicals
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40 13 on the enzymatic activity and kinetic parameters were carried out essentially by the
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43 14 standard methods with slight modifications as described above. The reaction mixture
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46 15 was centrifuged at 15,000 rpm for 3 min (MX-150; Tomy Seiko, Tokyo, Japan), and the
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49 16 supernatant was analyzed as described below.
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53 17 54 55 56 18 2.4. Analytical methods for enzyme activity 57 58 59 60 61 62 63 64 65

1 To evaluate the enzymatic activity in the transamination of β -phenylalanine with
2 2-oxoglutarate, the concentration of β -phenylalanine and benzoylactic acid in the
3 reaction mixture was analyzed by reverse-phase HPLC by using a Shimadzu LC-VP
4 system (Shimadzu, Kyoto, Japan) equipped with a Cosmosil 5C₁₈-AR-II column (0.46 ×
5 250 mm; Nacalai Tesque, Kyoto, Japan). The mobile phase was
6 acetonitrile/water/trifluoroacetate (4/6/0.1, v/v/v) at a flow rate of 1.0 ml/min and the
7 effluents were monitored at 210 nm by ultraviolet detection. One unit (U) of enzyme
8 activity was defined as the amount catalyzing the formation of 1 μ mol benzoylactic
9 acid per min from racemic β -phenylalanine under the above conditions. To investigate
10 the substrate specificity for the amino-acceptor, the decrease in β -phenylalanine
11 concentration by transamination with β -phenylalanine as an amino-donor and various
12 amino-acceptors was monitored by the same method. To investigate the substrate
13 specificity for the amino-donor, the formation of L-glutamate by transamination with
14 2-oxoglutarate as an amino-acceptor and various amino-donors was monitored using an
15 amino acid analyzing system consisting of Separations module 2695 (Waters, Milford,
16 MA, USA) and Multi λ fluorescence detector 2475 (Waters). Samples were analyzed
17 after derivatization with *N*-hydroxysuccinimidyl-6-aminoquinolinylcarbamate. The
18 effluents were monitored by fluorescence detection (excitation, 250 nm; emission, 395

1 nm).

2 Analysis of the enantiomeric purity of the remaining β -phenylalanine was performed
3 using reverse-phase HPLC after derivatization with
4 2,3,4,6-tetra-*o*-acetyl- β -D-glucopyranosyl isothiocyanate (GITC) [15-17].

5 Methanol/water (55/45, v/v, pH 2.5 adjusted with phosphoric acid) was used as the
6 mobile phase. Investigation of stereospecificity towards
7 3-amino-3-(3-pyridyl)propionate was also performed by the same method.

8 9 *2.5. Analytical methods for proteins*

10 Protein concentrations were determined using a protein assay kit (Bio-Rad, Hercules,
11 CA, USA) with bovine serum albumin as a standard [18]. To assay the relative
12 concentration of protein eluted by liquid chromatography, the effluents were monitored
13 at 280 nm by ultraviolet detection.

14 The relative molecular mass of the native enzyme was determined by gel-filtration
15 liquid chromatography. This method was carried out using a fast protein liquid
16 chromatography (FPLC) system (Amersham Pharmacia Biotech Co., Uppsala, Sweden)
17 equipped with Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech Co.).
18 The mobile phase comprised 200 mM NaCl and 20 mM potassium phosphate buffer

(pH 7.0) and the flow rate was 0.3 ml/min. Partially purified enzyme solution was injected and the eluate was fractionated at 0.3 ml/tube. The molecular mass of the enzyme was calculated based on the relative mobility using standard proteins (M.W. marker; Oriental Yeast Co., Tokyo, Japan).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 12.5% polyacrylamide gel using a Tris/glycine buffer system [19]. The molecular mass of the subunit of the enzyme was determined from a comparison of the mobility of the purified enzyme in SDS-PAGE with those of standard proteins (M.W. marker III; Daiichi Kagaku Yakuhin, Tokyo, Japan).

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2.6. Enzyme purification

All procedures were carried out at 0-5°C, and 20 mM potassium phosphate buffer (pH 7.0) containing 0.2 mM PLP was used as a standard buffer unless otherwise specified. *Variovorax* sp. JH2 cells (about 28 g obtained from 5 L of culture broth) were suspended in 100 ml of buffer and disrupted for 10 min using an ultrasonic oscillator (Kubota, Osaka, Japan). This sample was ultracentrifuged at 40,000 rpm for 1 h (L-80 Ultracentrifuge; Beckman, Fullerton, CA, USA) and the supernatant was used as a cell-free extract. The cell-free extract was fractionated with solid ammonium

1 sulfate. The precipitate at 30-50% saturation of ammonium sulfate was obtained by
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5 2 centrifugation at 12,000 rpm for 20 min (High Mac CR21; Hitachi, Tokyo, Japan) and
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8 3 dissolved in the same buffer. This enzyme solution (50 ml) was dialyzed against 3 L
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11 4 of buffer for 12 h and the dialyzed enzyme solution was applied to a DEAE-Sephacel
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14 5 column (3.0 × 7.0 cm; Amersham Pharmacia Biotech Co.) equilibrated with the same
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17 6 buffer. After washing the column with 200 ml of buffer, the enzyme was eluted with a
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20 7 linear gradient of 0-1.0 M NaCl in 250 ml buffer. The fractions containing the enzyme
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23 8 activity were combined (270 ml) and dialyzed for 12 h against 3 L of buffer. After the
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26 9 addition of 4 M NaCl, the enzyme solution was applied to a Phenyl-sepharose CL-4B
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29 10 column (3.0 × 7.3 cm; Amersham Pharmacia Biotech Co.) equilibrated with buffer
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32 11 containing 4 M NaCl. After washing the column with 500 ml of buffer, the enzyme
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35 12 was eluted with a linear gradient of 0-0.5% (v/v) Tween 20 in 200 ml of buffer. The
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38 13 fractions containing the enzyme activity were combined (125 ml) and dialyzed for 12 h
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41 14 against 3 L of buffer comprising 20 mM Tris/HCl buffer (pH 9.0) and 0.2 mM PLP.
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44 15 The dialyzed enzyme solution was concentrated to 10 ml by ultrafiltration using
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47 16 Centriprep YM-3 (MILLIPORE, Bedford, MA, USA). The enzyme solution was
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50 17 applied to a Mono Q HR 5/5 column (Amersham Pharmacia Biotech Co.) equilibrated
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53 18 with buffer comprising 20 mM Tris/HCl buffer (pH 9.0) and 0.2 mM PLP. After
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1 washing the column with the buffer, the enzyme was eluted with a linear gradient of
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5 2 0-1.0 M NaCl in 60 ml of the buffer. The fractions containing the enzyme activity
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9 3 were combined (4 ml) and dialyzed for 12 h against 2 L of buffer comprising 20 mM
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12 4 potassium phosphate buffer (pH 7.0) and 0.2 mM PLP. This sample was used as
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15 5 purified enzyme solution for characterization of its biochemical properties.
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21 7 *2.7. Preparation of internal peptides from the purified enzyme*
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24 8 Enzyme solution containing 1 mg of the purified enzyme was lyophilized and
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27 9 dissolved in 100 μ l of buffer comprising 20 mM Tris/HCl (pH 9.0) and 8 M urea.
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30 10 After incubation at 37°C for 60 min for denaturation, the concentration of urea was
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33 11 decreased to 4 M by adding 100 μ l of 20 mM Tris/HCl buffer (pH 9.0). After the
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36 12 addition of 0.05 nmol lysyl endopeptidase (Wako Pure Chemicals, Osaka, Japan), the
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39 13 solution was incubated at 30°C for 6 h for digestion. The digested sample was applied
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42 14 to a μ RPC C2/18 SC2/10 column (Amersham Pharmacia Biotech Co.) connected to a
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45 15 SMART system (Amersham Pharmacia Biotech Co.) and equilibrated with mobile
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48 16 phase of 0.1% (v/v) trifluoroacetic acid in water. The peptides were eluted using a
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51 17 linear gradient of 0-80% (v/v) acetonitrile in 10 ml of mobile phase at a flow rate of 0.1
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54 18 ml/min. The amino acid sequences of the internal peptides were analyzed by
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1 automated Edman degradation with a 491HT protein sequencer (Applied Biosystems,
2 Foster City, CA, USA).

3. Results

3.1. Purification of (S)-BAT

(S)-BAT was purified about 17-fold by four successive steps from the cell-free extract of *Variovorax* sp. JH2. Typical results of the purification are shown in Table 1. The final preparation gave a single band on SDS-PAGE (Fig. 1) and showed specific activity of 1.5 U/mg. The subunit molecular mass of purified enzyme determined by SDS-PAGE was about 40,000 (Fig. 1). The native molecular mass determined by gel-filtration chromatography was about 72,000.

3.2. Kinetic parameters of (S)-BAT

Kinetic parameters of (S)-BAT in the transamination reaction with racemic β -phenylalanine and 2-oxoglutarate were investigated as described in Materials and methods except that the reaction was performed with a variety of substrate concentrations. The K_m value for racemic β -phenylalanine in the reaction with 10 mM 2-oxoglutarate was 0.63 mM, and the V_{max} value was 1.7 U/mg. The K_m value for

1 2-oxoglutarate in the reaction with 10 mM racemic β -phenylalanine was 2.5 mM.

2

3 3.3. Stereoselectivity of (S)-BAT

4 To investigate the stereoselectivity of (S)-BAT in the transamination of racemic

5 β -phenylalanine with 2-oxoglutarate for the enantioselective production of

6 (R)- β -phenylalanine from racemic β -phenylalanine, the time-course of the reaction was

7 analyzed essentially by the standard methods (Fig. 2). In a 1 h reaction, half of the 10

8 mM racemic β -phenylalanine was decomposed and about 5 mM benzoylacetic acid was

9 produced. The optical purity of the remaining β -phenylalanine reached 99.6% *e.e.* for

10 (R)- β -phenylalanine. Longer incubation did not result in a further decrease in the

11 amount of β -phenylalanine. This result indicated that the purified enzyme catalyzed

12 (S)- β -phenylalanine-specific transamination. In the longer incubation, benzoylacetic

13 acid was degraded gradually by spontaneous decarboxylation.

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15 3.4. Substrate specificity of (S)-BAT

16 The substrate specificity of (S)-BAT was investigated (Table 2). As amino-donors,

17 10 mM β -phenylalanine analogs, some other β -amino acids, proteinogenic amino acids,

18 and their D-isomers were tested with 10 mM 2-oxoglutarate as an amino-acceptor.

Besides β -phenylalanine, 3-amino-3-(3-pyridyl)propionate served as a good substrate for the enzyme and the relative activity was 56% of the enzymatic activity for racemic β -phenylalanine. In the enantiomer analysis of the remaining amino acid during racemic 3-amino-3-(3-pyridyl)propionate deamination, a decrease in only one enantiomer was observed on HPLC analysis after GITC derivatization; indicating that the enzyme had stereoselectivity for 3-amino-3-(3-pyridyl)propionate.

Extremely low activity was observed with L-glutamine, L-aspartate, L-asparagine, β -alanine, and L-alanine, and the relative activities for these compounds were 1.1%, 0.57%, 0.30%, 0.28%, and 0.07% of the enzymatic activity for racemic β -phenylalanine, respectively. The following compounds were judged to be inactive as amino-donors: other proteinogenic amino acids (L-proline and L-glutamate were not tested), D-isomers of 18 proteinogenic amino acids (D-proline and D-glutamate were not tested), DL- β -aminoisobutyrate, taurine, L-phenylglycine, D-phenylglycine, L-homophenylalanine, D-homophenylalanine, (*R*)-2-amino-2-phenylethanol, (*S*)-2-amino-2-phenylethanol, (*R*)-1-phenylethylamine, (*S*)-1-phenylethylamine, (*R*)-1-(1-naphthyl)ethylamine, (*S*)-1-(1-naphthyl)ethylamine, and, 1,2,3,4-tetrahydro-1-naphthylamine.

As an amino-acceptor, various oxo acids and ketones were tested at concentrations of

10 mM with 10 mM racemic β -phenylalanine as the amino-donor. Oxaloacetate, pyruvate, and 1,3-acetonedicarboxylate served as the amino-acceptors. The relative activities were 9.9%, 3.8%, and 2.0% of the enzymatic activity for 2-oxoglutarate, respectively. The following compounds were judged to be inactive as amino-acceptors: 3-phenylpyruvate, 2-oxovarelate, ketomalonate, 4-oxovalerate, benzoylacetate, 2-heptanone, acetophenone, and ethyl benzoylacetate.

3.5. Effects of pH and temperature on the activity and stability of the enzyme

The optimum pH for the enzymatic activity (β -phenylalanine:2-oxoglutarate transamination) was measured at 28°C under various pH conditions. The enzyme showed a maximum activity at pH 9.5 (Fig. 3A). The optimum temperature for the enzymatic activity was assayed at pH 9.0 at various temperatures (0-65°C). The enzyme activity was found to be maximally active at 50°C (Fig. 3B).

The enzyme was incubated at 28°C for 30 min under various pH conditions, and then a sample of the enzyme solution was taken and the residual activity (β -phenylalanine:2-oxoglutarate transamination) was measured. The enzyme was stable in the pH range 6.0-8.0 (Fig. 4A). The enzyme was preincubated for 30 min at pH 9.0 under various temperatures (0-65°C) and the residual activity was assayed.

The enzyme was inactivated entirely by the incubation above 60°C (Fig. 4B).

3.6. Effects of inhibitors and metal ions on the enzymatic activity

The effects of various inhibitors and metal ions (2 mM) on the enzyme activity were examined by the standard methods. The enzyme activity was inhibited by Ag^+ , Hg^{2+} , Zn^{2+} , dinitrophenol, and hydroxylamine and decreased to 0%, 2.4%, 52%, 46%, and 23%, respectively, of the original activity. The other metal ions tested (Li^+ , Na^+ , K^+ , Rb^+ , Cs^+ , Be^{2+} , Mg^{2+} , Ca^{2+} , Sr^{2+} , Ba^{2+} , Fe^{2+} , Co^{2+} , Ni^{2+} , Sn^{2+} , Pb^{2+} , Fe^{3+} , Al^{3+} , and Cr^{3+}) were judged not to affect the enzymatic activity. Metal ion chelaters (ethylenediamine-*N,N,N',N'*-tetraacetic acid, 8-hydroxyquinoline, and *o*-phenanthroline), serine protease inhibitors (phenylmethane sulfonylfluoride and diisopropylphosphofluoride), and sulfhydryl inhibitors (5,5'-dithiobis-2-nitrobenzoate, iodoacetate, *p*-chloromercuribenzoate, and *N*-ethylmaleimide) were judged to have no significant influence on the activity.

3.7. Analysis of amino acid sequences

The amino acid sequences of internal peptides were analyzed and three parts of the amino acid sequence were determined. These amino acid sequences were named IP1

(54 residues), IP2 (67 residues), and IP3 (63 residues) and were applied to homology searches using BLAST (Fig. 5). All three amino acid sequences showed homology with aminotransferases and, especially, β -aminotransferases and glutamate-1-semialdehyde 2,1-aminomutases were most homologous with them [20-23].

4. Discussion

As a result of the present investigation of substrate specificity, β -phenylalanine and 3-amino-3-(3-pyridyl)propionate were found to serve as good amino-donors for the (*S*)-BAT-catalyzed reaction. The enzyme catalyzed transamination with the other amino-donors such as L-glutamine, L-aspartate, L-asparagine, β -alanine, and L-alanine, but the activities with these compounds were extremely low. These findings suggested that the enzyme recognizes the 3-amino-3-aromatic propionate-skeleton specifically. This result also indicated that the enzyme is different from already-known ω -aminotransferases or aromatic aminotransferases. With regard to stereoselectivity for amino-donors, the enzyme was found to catalyze (*S*)-enantiomer selective transamination in the reactions with racemic β -phenylalanine and also showed enantioselectivity in transamination with racemic 3-amino-3-(3-pyridyl)propionate.

1 Concerning the amino-acceptor, several oxo acids, such as 2-oxoglutarate, pyruvate,
2 oxaloacetate and 1,3-acetonedicarboxylate, served as substrates. In particular,
3 2-oxoglutarate was found to be the most suitable substrate as the amino-acceptor.
4 There are several differences between the (S)-BAT from *Variovorax* sp. JH2 and
5 β -aminotransferases from *Mesorhizobium* sp. in terms of substrate specificity. The
6 β -aminotransferases of *Mesorhizobium* sp. acted on some amino acids that are not the
7 substrate of (S)-BAT, such as β -alkyl- β -amino acids, aromatic α -amino acids
8 (phenylalanine and tyrosine), aliphatic α -amino acids (valine and leucine) and also acted
9 on arylamines, such as 1-phenylethylamine. Furthermore, both 2-oxoglutarate and
10 pyruvate have been reported to serve as good amino-acceptors for β -aminotransferases
11 from *Mesorhizobium* sp. These results indicated that β -aminotransferases from
12 *Mesorhizobium* sp. is an intermediate enzyme between β -aminotransferases,
13 ω -aminotransferases, and α -amino acid aminotransferases. These facts led to the
14 conclusion that the (S)-BAT from *Variovorax* sp. JH2 is a novel aminotransferase highly
15 specific to β -aryl- β -amino acids with preference for 2-oxoglutarate as an
16 amino-acceptor.

17 As the result of homology searches of the amino acid sequence, (S)-BAT showed the
18 highest homology with glutamate 1-semialdehyde 2,1-aminomutases as well as β -

1 aminotransferases [6]. These enzymes were classified into aminotransferase subgroup
2 II, containing acetylornithine, ornithine, ω -amino acid, 4-aminobutyric acid, and
3 diaminopelargonate aminotransferases, and it is possible that (S)-BAT is classified as a
4 member of the subgroup [24, 25]. It was reported that glutamate 1-semialdehyde
5 2,1-aminomutases act in porphyrin and chlorophyll metabolism in vivo, however,
6 (S)-BAT acts in β -phenylalanine assimilation of *Variovorax* sp. JH2 [25, 26]. In the
7 β -aminotransferases of *Mesorhizobium* sp., Arg43 and Arg404 supposed to be important
8 residues to recognize a carboxylate group [6]; however, the corresponding arginine
9 residues are not found in (S)-BAT (Fig. 5), which indicates the difference in these
10 enzymes.

11 Above all, it was clarified that (S)-BAT has a unique primary structure in
12 aminotransferase subgroup II and useful for optically active β -aryl- β -amino acid
13 production from racemic substrates by decomposition of specific enantiomer through
14 transamination in the presence of 2-oxoglutarate.

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Figure legends

Fig. 1 SDS-PAGE of the purified enzyme. Lane A is 1 mg of the purified enzyme.

Lane B is molecular mass standards (from top): phosphorylase *b* (97 kDa), BSA (66 kDa), aldolase (42 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa), and lysozyme (14 kDa).

Fig. 2 Time-course of the transamination with purified (*S*)-BAT. Concentrations of β -phenylalanine (BPA) (\square) and benzoylactic acid (\bullet) are shown. Optical purity of β -phenylalanine in the 1 h reaction is shown with an arrow.

Fig. 3 Effects of pH and temperature on the activity of (*S*)-BAT. The effects of pH (A) and temperature (B) on the enzymatic activity (β -phenylalanine:2-oxoglutarate transamination) are shown. Buffers (200 mM) used for pH adjustment are as follows: acetate/sodium acetate buffer (\square) for pH 5.0-6.0, potassium phosphate buffer (\blacktriangle) for pH 6.0-8.0, Tris/HCl buffer (\bullet) for pH 7.5-10.0, borate/NaOH buffer (\circ) for pH 10.0-10.5, Na_2PO_4 /NaOH buffer (\blacksquare) for pH 10.5-12.0.

Fig. 4 Effects of pH and temperature on the stability of (S)-BAT. The effects of pH (A) and temperature (B) on the stability are shown. Buffers (0.5 M) used for pH adjustment are as follows: acetate/sodium acetate buffer (□) for pH 4.0-6.0, potassium phosphate buffer (▲) for pH 6.0-8.0, Tris/HCl buffer (●) for pH 8.0-10.0, borate/NaOH buffer (○) for pH 9.0-11.0, Na₂PO₄/NaOH buffer (■) for pH 11.0-12.0.

Fig. 5 Amino acid sequence alignments of IP1-3 and proteins homologous to them. Identical amino acids to those in the sequences of IP1-3 are marked in gray. MES, β -aminotransferases from *Mesorhizobium* sp.; CNE, glutamate-1-semialdehyde 2,1-aminomutase from *Cryptococcus neoformans*; SPO, glutamate-1-semialdehyde 2,1-aminomutase from *Schizosaccharomyces pombe*; BHA, glutamate-1-semialdehyde 2,1-aminomutase from *Bacillus halodurans*; FRA, aminotransferase class-III from *Frankia* sp.; CDI, glutamate-1-semialdehyde 2,1-aminomutase from *Corynebacterium diphtheriae*.

A

B

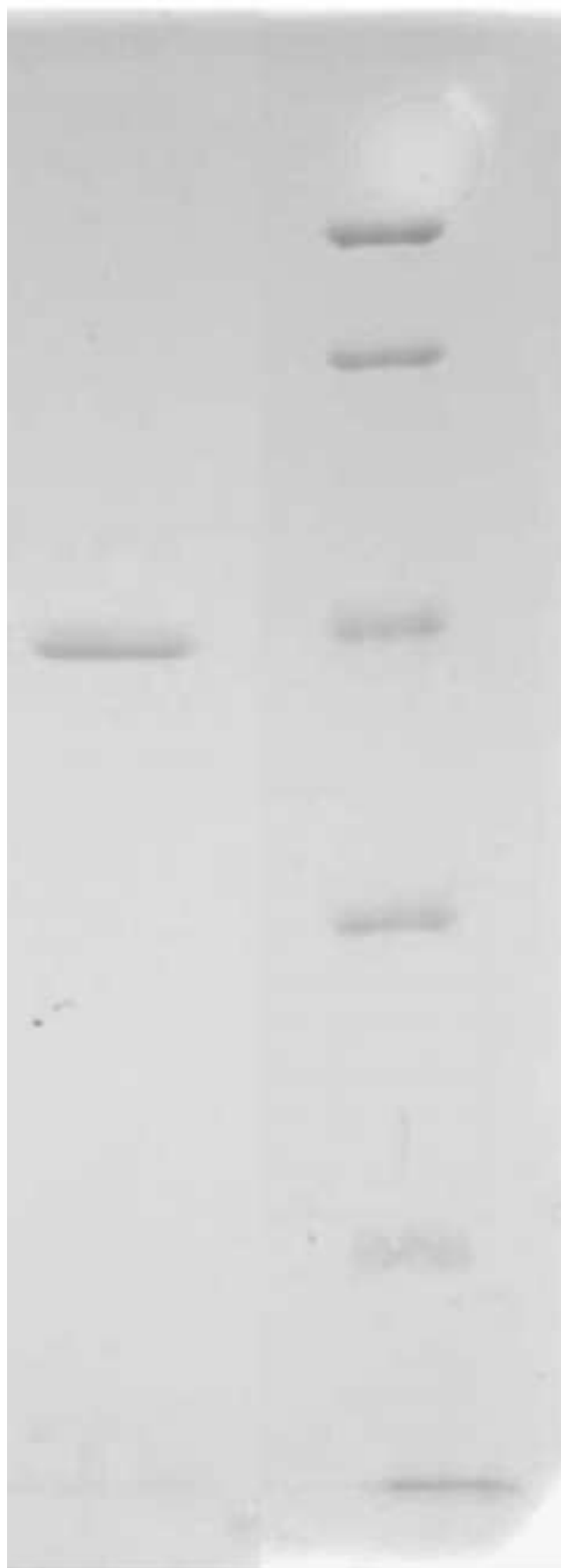


Figure2

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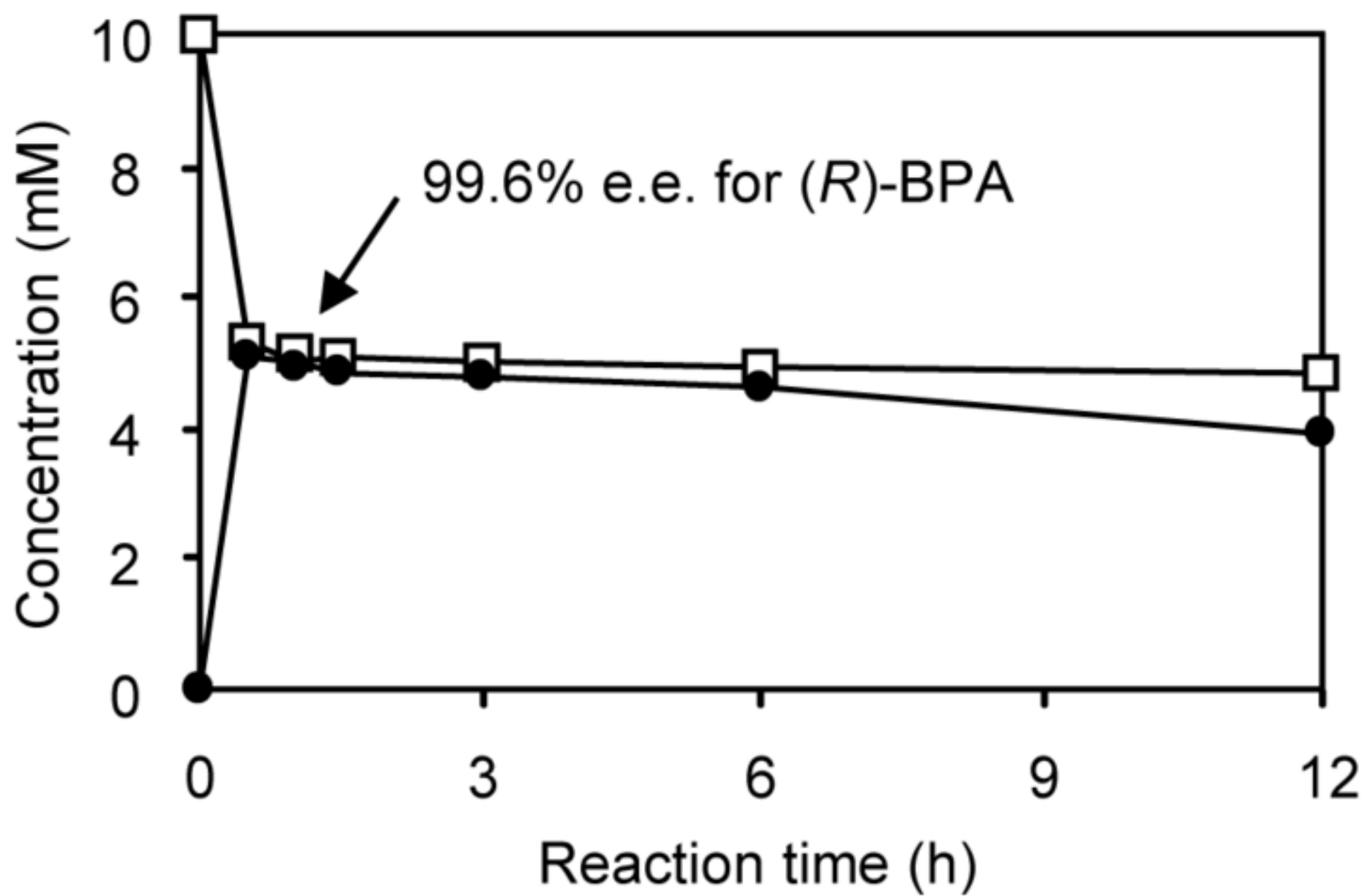


Figure3
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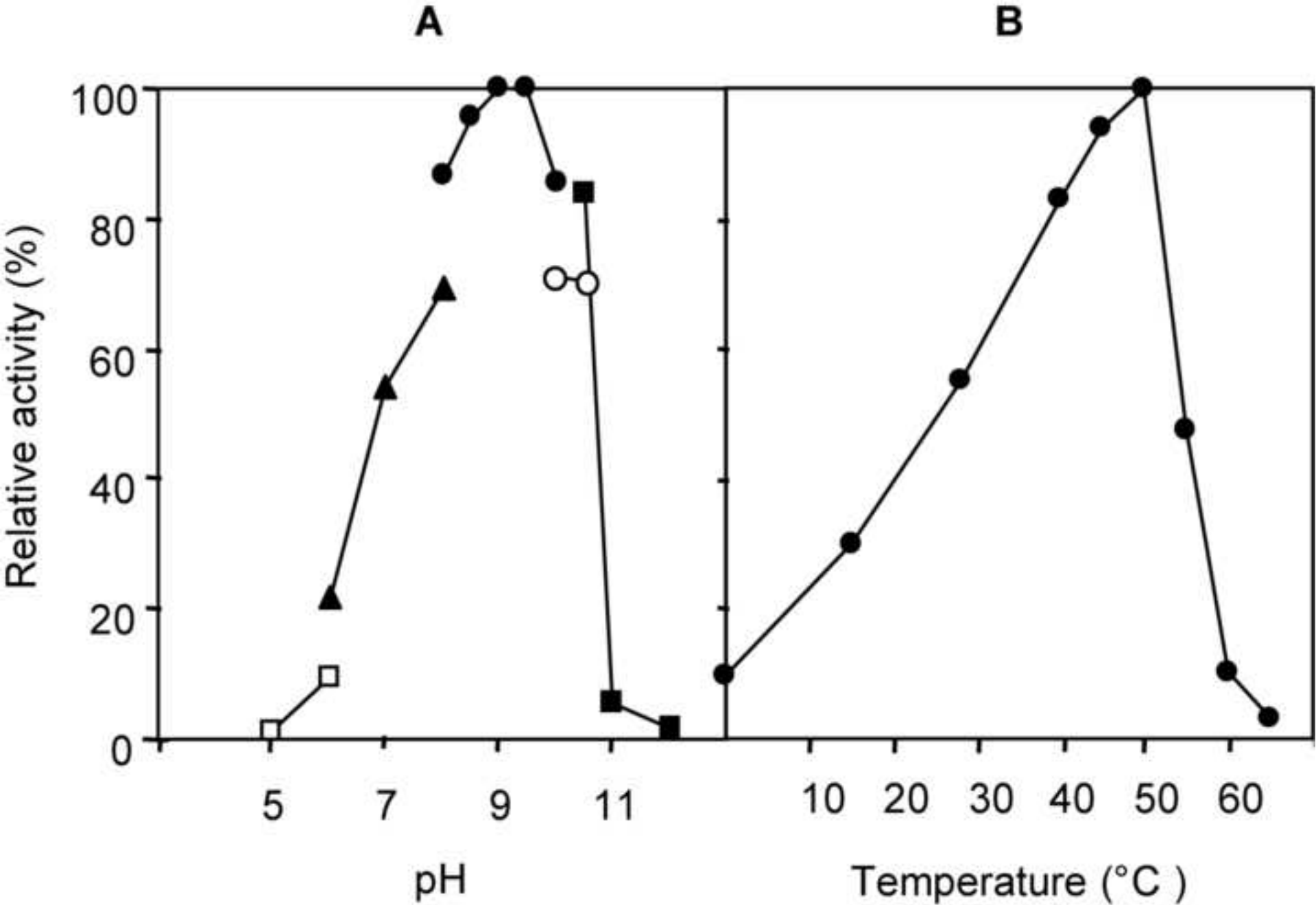


Figure4
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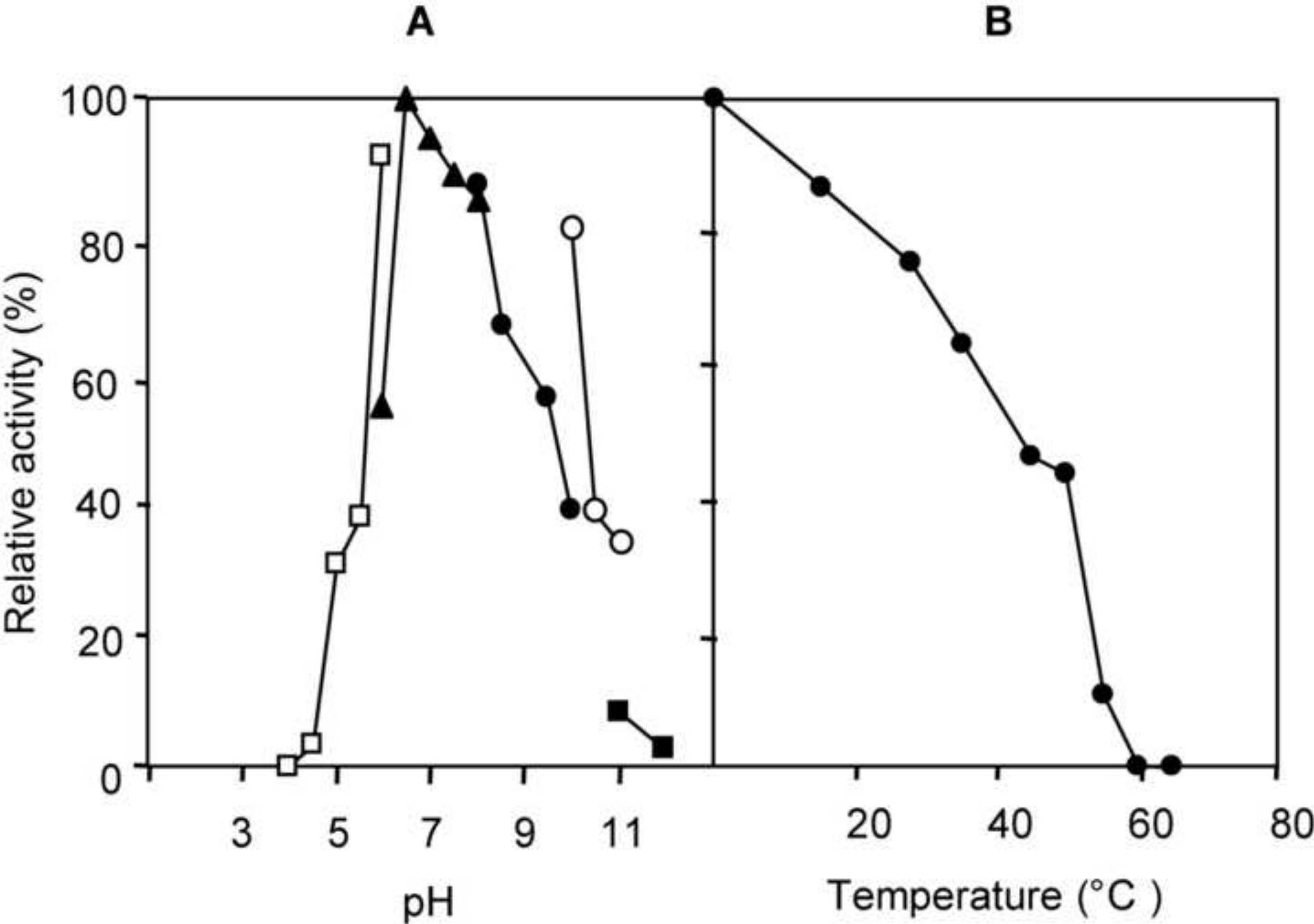


Figure5

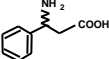
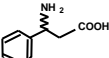
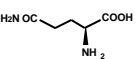
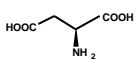
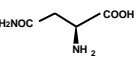


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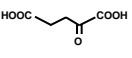
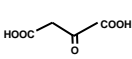

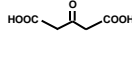
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	MES	15	D T A E K A Q A I A A A R N T F A R D N P V S A G H H E R A R R S M P G G N T R	54
	CNE	-1	- M T S L T Q A L K R A Q Q T Y A N R N R K S Y E A H Q A A S R H L P G G S T R	39
	SPO	4	E I S D L S L A K E K S E A Q F I A R N P K S N D F H C K A C D S L P G G N T R	44
	BHA	-3	- - - M A M V E D T R P E H T Y A E Q T P R S K S W I E Q A K K V M P G G I T A	37
	IP1	41	S V L F Y A P P P L T I A R	54
	MES	55	S I L F H R P P P L V I A Q	68
	CNE	40	S S I F I H P P P L C I Q K	53
	SPO	45	T V L H G A P P P I F I E A	58
	BHA	38	N I K H F A F Y F I V M K K	51
B	IP2	1	Y I G G G M S F G A F G G R A D V M A Q F D P R T - E A L A H S G T E N N N V M	39
	MES	281	Y I G G G M S F G A F G G R R D L M E R F D P A R D G A F A H A G T F N N N I L	320
	SPO	271	Y L G G G L P F G A F G G R A D I M S C F D P R L P G S L S H S G T F N N D T L	310
	CNE	269	F F G G G F A F G A F G G K K E I M E K F D A R K G G A I S H G G T F N N S P L	308
	FRA	296	A L G G G L P S G A I G A T A E L M E A V A S D R - - - V K Q V G T Y N G N P L	332
	IP2	40	T M A A G Q A G L T R L F T P E A A G X L X E R G X A M	67
	MES	321	T M S A G H A A L T Q I Y T R Q A A S D L S A S G D R F	348
	SPO	311	T L T A G Y V G L T E L Y T P E A V K R L N A L G D G L	338
	CNE	309	T M V A G A T A M E K I L T E D A L K N L N K L G D W M	336
	FRA	333	T M A A A R A S L F E V L T P D A Y T H L D R L G G R L	360
C	IP3	1	E G V A L Q F T G V G S L M N A H F L Q - G E V R R V A D L A A V D G R L R Q L	39
	MES	359	N Q A P L Q F T G L G S L G T I H F S R - A P I R S A G D V R A A D Q Q L K E L	397
	BHA	377	K G V P M K A V G I G S I F S V V L T E - E D I F N Y R D L O A S D L Q T R K L	415
	SPO	349	- - T K M S I T G L G S I M N I H F T E S G R V N S Y N D T A G E V I E L K D L	386
	CDI	343	E G V A H H I Q R A A T M L S V R F A E - G E G H N F D D M K A A D T F R F P A	381
	IP3	40	L F F H L L N A G I Y T S P R G F V V L S L P P	63
	MES	398	F F F H M L R K G I Y L A P R G M Y A L S L E I	421
	BHA	416	I D L H L L N E G I Y T K P - - L N R Y S L S T	437
	SPO	387	L W M D L L K E G F W I A R R G M I T L S L V L	410
	CDI	382	F F H A L L D N G I Y A S P S V F E T W F V S A	405

Table 1. Purification of (*S*)-BAT

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Cell-free extract	1260	109	0.087	100
Ammonium sulfate fractionation	410	83	0.20	76
DEAE sephacel	170	42	0.24	38
Phenyl sepharose	4.0	3.4	0.85	3.1
Mono Q HR 5/5	0.1	0.14	1.5	0.13

Table 2. Substrate specificity of (*S*)-BAT for amino donor (A) and for amino acceptor (B)

(A)		
Amino donor	Structural formula	Relative activity (%)
Racemic BPA		100
Racemic 3-amino-3-(3-pyridyl)propionic acid		56.0
L-Glutamine		1.1
L-Aspartate		0.57
L-Asparagine		0.30
β-Alanine		0.28
L-Alanine		0.07

(B)		
Amino acceptor	Structural formula	Relative activity (%)
2-Oxoglutarate		100
Oxaloacetate		9.9
Pyruvate		3.8
1,3-Acetonedicarboxylate		2.0